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TURNOVER OF ACETYLCHOLINE RECEPTORS: MECHANISMS OF REGULATION

ANNUAL REPORT

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The synthesis, insertion and degradation of acetylcholine receptors (AChRs) of skeletal muscle cells are closely regulated both by the muscle cells and by the motor nerves that supply them. The goal of this project is to elucidate the mechanisms of regulation of the AChRs, both at the neuromuscular junction and at extrajunctional regions.

We have used ^{125}I-α-bungarotoxin (^{125}I-α-BuTx) as a label to follow the metabolic turnover of AChRs both in vivo and in tissue culture. We have used complementary DNAs (cDNAs) that specifically bind to the messenger RNA (mRNA) for subunits of the AChR to measure the appropriate mRNAs (gene regulation), as well.

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Trophic control
Receptor turnover
Messenger RNA
Receptor stabilization
Synaptic transmission

19. Abstract (continued)

During the past year, our accomplishments have included the following:

1) Completion of studies showing that AChRs are synthesized and inserted rapidly at the neuromuscular junction.

2) Completion of studies and a manuscript indicating that the motor nerve is essential for stabilization of the "rapidly turned over AChRs" (RTOs) at the neuromuscular junction.

3) We are developing a mathematical model to describe the kinetics of synthesis, degradation, and stabilization of junctional AChRs.

4) We have acquired, prepared, and labeled cDNA probes for the α- and gamma subunits of the AChR, for use in the studies of neural regulation of AChR synthesis.

5) We have shown that presynaptic blockade of quantal acetylcholine (ACh) transmission results in a denervation-like increase of messenger RNA (mRNA) for the α-subunit of the acetylcholine receptor.

6) We have developed a method for blockade of the mouse and rat soleus muscles, using implantable osmotic pumps to deliver the postsynaptic blocking agent α-BuTx.

7) We have shown that maintenance of stability of junctional AChRs requires neuromuscular transmission of ACh:

a) Blockade of quantal ACh transmission by means of botulinum toxin results in a denervation-like acceleration of degradation of junctional AChRs.

b) Postsynaptic blockade of both quantal and non-quantal ACh transmission, using α-BuTx, produces acceleration of degradation of junctional AChRs that is quantitatively equivalent to the effect of denervation, with an identical time course.

Foreword

Citations of commercial organizations and trade names in this report do not constitute an official Department of the Army endorsement or approval of the products or services of these organizations.

In conducting the research described in this report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 86-23, Revised 1985).

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I. Introduction and Summary of Research Project

1. Introduction:

The major goal of this investigation is to learn more about the mechanisms that regulate the synthesis and turnover of junctional and extrajunctional acetylcholine receptors (AChRs). During the past year, we have made significant progress in many aspects of these studies. Our findings, detailed in Section II (Progress), indicate the following:

1) Junctional AChRs are rapidly synthesized. The rapidly turned over junctional AChRs (RTOs) are precursors of the stable junctional AChRs (Drachman et al., 1987). These findings have been described in previous reports.

2) The motor nerve plays a key role in "stabilizing" a proportion of the newly inserted RTOs, converting them to the stable form. This work has been completed.

3) A mathematical model is being developed to describe the above phenomena, including:

a) Degradation of 2 classes of junctional AChRs - RTOs and stable receptors

b) Rapid synthesis and insertion of AChRs

c) The process of stabilization of a proportion of new RTOs at the neuromuscular junction, which is dependent upon the motor nerve.

4) Maintenance of stability of junctional AChRs requires acetylcholine (ACh) transmission. Pharmacological blockade of quantal ACh transmission, using botulinum toxin, results in a denervation-like accelerated loss of stable junctional AChRs. However, this effect begins later than the effect of denervation.

Pharmacological blockade of quantal plus non-quantal ACh transmission, using α-bungarotoxin (α-BuTx), results in accelerated loss of stable junctional AChRs; this rate of loss is quantitatively equivalent to that of surgical denervation.

5) We have completed studies showing that ACh transmission plays a key role in regulating transcription of mRNA for the α-subunit of AChR. Blockade of quantal transmission with botulinum toxin produces a denervation-like increase in levels of messenger RNA (mRNA) for the α-subunit of AChR.

6) We have acquired a complementary DNA (cDNA) probe for the gamma-subunit of AChR, and are using both the α and gamma cDNAs in studies of neural regulation of mRNAs for AChR.

2. Brief Restatement of Overall Research Problem and Rationale:

Both the distribution and turnover of acetylcholine receptors (AChRs) of mammalian skeletal muscles are regulated to a large extent by the motor nerves. In innervated muscles, AChRs are localized almost exclusively at neuromuscular junctions (Axelsson and Thesleff, 1959; Miledi, 1960, Albuquerque et al., 1974; Fertuck and Salpeter, 1974; Kuffler and Yoshikami, 1975). Following denervation, a great increase of extrajunctional AChRs occurs (Miledi, 1960; Miledi and Potter, 1971; Lee, 1972; Chang et al., 1975; Hartzell and Fambrough, 1972; Pestronk et al., 1976a,b). This has been attributed to increased synthesis of AChRs, on the basis of studies of the rate of appearance of completed receptor molecules (Fambrough, 1970; Grampp et al., 1972; Brockes and Hall, 1975b; Devreotes and Fambrough, 1976). Recent studies have shown that the levels of mRNAs corresponding to the AChR subunits increase rapidly after denervation, presumably as a result of increased transcription of the appropriate mRNAs (Merlie et al., 1984; Goldman et al., 1985; Evans et al., 1987; Moss et al., 1987; Shieh et al., 1987; Parker et al., 1988).

Junctional ACh Receptors: AChRs of neuromuscular junctions differ in many respects from extrajunctional AChRs. They are densely packed, located mainly at the peaks of post-junctional folds (Mathews-Bellinger and Salpeter, 1978). They differ physiologically, physicochemically and immunologically from extrajunctional AChRs (Brockes and Hall, 1975a; Lindstrom et al., 1976; Neher and Sakmann, 1976; Sakmann and Brenner, 1978; Schuetze and Fishbach, 1978; Weinberg and Hall, 1979; Dwyer et al., 1981; Brenner and Sakmann, 1983).

One of the most important characteristics of junctional AChRs, which is a major focus of this research, is their metabolic stability, with a half-life previously reported to be between 6 and 13 days (in rodents) (Berg and Hall, 1975; Chang and Huang, 1975; Stanley and Drachman, 1978; Linden and Fambrough, 1979; Bevan and Steinbach, 1983). We have reported that the AChRs at innervated neuromuscular junctions actually comprise two subpopulations with strikingly different rates of turnover (Stanley and Drachman, 1983a; 1987). The majority of junctional AChRs are stable, with a half-life of 11 to 12 days. The remainder, which we now estimate to be 20 to 25% of the total, are RTOs with a half-life of approximately 1 day. This finding is based on our detailed analyses of degradation curves of ^{125}I-labeled AChRs, using an in vivo mouse model (Stanley and Drachman 1983a, 1987). This result, which we have repeatedly confirmed in the course of our subsequent studies described below, leads to several conclusions and predictions:

1) It predicts that the rate of synthesis and insertion of junctional AChRs should be more rapid than previously estimated, in order to replace the rapidly degraded AChRs.

2) This may explain the rapid recovery from certain neuroparalytic toxins. Recovery from irreversible AChR blocking agents (such as α-bungarotoxin α-BuTx) is known to occur far more quickly than would be expected on the basis of dissociation of the toxin (Fertuck et al., 1975; Pestronk and Drachman, 1978).

3) It suggests that the turnover of RTOs alone accounts for the majority of the overall junctional receptor turnover. Although the population of RTOs is only 20 to 25% of the total AChR population, its rate of turnover is

10 times as rapid as the rate for stable AChRs.

4) Perhaps most significant, the RTO subpopulation of junctional AChRs appear to be precursors of the stable AChRs.

Our findings strongly support:

1) The rapid synthesis of junctional AChRs (Ramsay et al., 1988).
2) The concept that the RTOs are converted to stable AChRs (Stanley and Drachman, 1987; Ramsay and Drachman, submitted).

Neural Control of Junctional AChRs: There is abundant evidence that many of the properties of junctional AChRs are regulated to a large extent by the motor nerves (Salpeter and Loring, 1985; Schuetze and Role, 1987). For example, the ionic channel properties of short open times and high ionic conductances are dependent on motor innervation (Neher and Sakmann, 1976; Sakmann and Brenner, 1978; Schuetze and Fishbach, 1978; Sellin and Thesleff, 1981; Brenner and Sakmann, 1983). Clustering of AChRs occurs at the site of contact between the motor nerve endings and the muscle cell membrane (Takeuchi, 1963; Anderson and Cohen, 1977; Bevan and Steinbach, 1977; Burden, 1977; Reiness and Weinberg, 1981). Some nerve-induced modification of the membrane (possibly the basement membrane) is thought to determine the high density accumulation of AChRs at this site (Burden et al., 1979). Stability of junctional AChRs is also dependent on the motor nerve. Both the initial appearance of stable AChRs during development (Burden, 1977; Reiness and Weinberg, 1981) and the continued maintenance of AChR stability (Chang and Huang, 1975; Bevan and Steinbach, 1977; Brett and Younkin, 1979; Levitt and Salpeter, 1981; Stanley and Drachman, 1981) require some influence of the motor nerve.

We have shown that the motor nerve plays an essential role in stabilizing a proportion of the RTOs junctional AChRs, converting them to the stable form. During the past year, we have completed a manuscript describing these results, and have submitted it for publication (Ramsay and Drachman, submitted).

Little is presently known about the mechanisms by which these changes in junctional AChRs are brought about. There is some evidence that the initial localization of the nerve-muscle junction and clustering of AChRs involve nerve-muscle contact, rather than neurotransmission (Steinbach et al., 1975). On the other hand, normal channel properties of junctional AChRs may require ACh transmission (Sellin and Thesleff, 1981). One of the hypotheses in the present project is that ACh transmission mediates the nerve's effect on the stabilization of AChRs at the neuromuscular junction.

We now have now completed studies showing that AChR transmission plays a key role in the maintenance of stability of junctional AChRs (see Progress, Section II, Task 7; Avila et al., submitted).

Extrajunctional ACh Receptors: In normally innervated muscles, the density of AChRs at extrajunctional regions is very low -- typically less than 20 α-BuTx binding sites per μm^2 for the soleus muscles of rodents (Pestronk et al, 1976a,b; Fambrough, 1979). However, the density of extrajunctional AChRs is increased in skeletal muscle cells that lack innervation (immature, or denervated, mature muscle) (Axelsson and Thesleff, 1959; Miledi, 1960; Miledi

and Potter 1971; Diamond and Miledi, 1962; Dryden, 1970; Fambrough and Rash, 1971; Lee, 1972; Letinsky, 1975; Pestronk et al., 1976a,b; Bevan and Steinbach, 1977; Drachman et al., 1984). The high density of extrajunctional AChRs in these situations is thought to be due to a high rate of receptor synthesis (Fambrough, 1970; Grampp et al., 1972; Brockes and Hall, 1975b; Devreotes and Fambrough, 1976). Recent evidence indicates that there is a high rate of transcription of the genes for the various subunits of AChR, resulting in increased amounts of the appropriate mRNAs in denervated muscle (Merlie et al., 1984; Goldman et al., 1985; Klarsfeld and Changeux, 1985; Evans et al., 1987; Moss et al., 1987; Shieh et al., 1987). cDNA probes are now available for these mRNAs (Merlie et al., 1983; LaPolla et al., 1984; Boulter et al., 1985).

We have acquired cDNA probes for the α- and gamma-subunits, and are using them as sensitive and particularly relevant probes in our studies of neural regulation of AChR synthesis (see Section II Task 4a).

II. Progress (8/1/87 - 11/30/88)

Overall progress:

During the past year we have continued to make progress in our studies of regulation of junctional and extrajunctional AChRs in skeletal muscle. We have completed studies supporting several of the central features of our hypothesis that ACh transmission plays a key role in the neural regulation of both junctional and extrajunctional AChRs. Furthermore, we are applying methods of pharmacology and molecular biology, which are yielding important information regarding the fundamental goals of this project.

Task 1. To determine the time course of new AChR synthesis and insertion:

This project was completed last year. A paper describing the results has been published (Ramsay et al., 1988). The results of the study showed that AChRs are synthesized and inserted at neuromuscular junctions at a rapid rate. This finding is consistent with our previous conclusion that the normal innervated neuromuscular junction contains a subpopulation of RTOs.

A mathematical model describing the kinetics of turnover of junctional AChRs is now in progress (see below).

Task 2. To determine whether denervation prevents post-insertional stabilization of junctional AChRs:

This study has been completed, and the findings described (Drachman et al., 1987). Our results showed that AChRs are initially inserted at neuromuscular junctions in an unstable form (RTOs). Stabilization of these receptors requires the presence of the nerve. Surgical denervation prevents the newly inserted AChRs from being stabilized.

During the past year, we completed a manuscript describing these results, and have submitted it for publication (Ramsay, submitted).

Mathematical Model of Kinetics of Junctional Receptor Turnover and

Stabilization: As indicated above, we have now completed a series of experimental studies describing the synthesis, degradation, and stabilization of AChRs at neuromuscular junctions in the mouse sternomastoid muscle. These results provide a coherent picture of the metabolism of postsynaptic receptors at a normally innervated model synapse, i.e., the neuromuscular junction. They are sufficiently consistent to permit us to construct a mathematical model describing these phenomena. I therefore have undertaken this project in collaboration with Dr. Richard Drachman, a mathematical physicist at the Goddard National Aeronautics and Space Administration Laboratories.

A series of differential equations have been derived from the following considerations:

1) The total number of AChRs at the neuromuscular junction remains constant, and is noted as C_N.

a) In normally innervated junctions, the number of RTOs (R) and stable AChRs (S) each remains constant, noted as C_R and C_S.

2) The total is made up of labeled RTOs (R*) and unlabeled RTOs (R) plus labeled stable AChRs (S*) and unlabeled stable AChRs (S).

3) Each of these populations is degraded at its own rate, λ_R and λ_S, which is given in units of day^{-1}.

4) In addition, a proportion of RTOs (R) are converted to stable AChRs (S). These are detected in the experiment as R* becoming S*.

5) In view of steady-state considerations (1, above), the number of (R + R*) that are converted to (S + S*) is just sufficient to replace the (S + S*) that are lost through degradation.

The decay equation derived from the above considerations is as follows:

$$N_R^* + N_S^* = N_{(t)}^* = \left[C_R + \frac{\lambda_S}{\lambda_S - \lambda_R} \cdot C_S\right] e^{-L_R \cdot t} + C_S \left[\frac{L_R}{L_R - \lambda_S}\right] e^{-\lambda_S \cdot t}$$

where:

NR^* = the number of labeled RTOs

NS^* = the number of labeled stable AChRs

N^* = the total number of labeled AChRs at time t

CR = the constant number of RTOs (labeled + unlabeled).

C_s = the constant number of stable AChRs (labeled + unlabeled)

λ_R = the rate of degradation of RTOs

λ_S = the rate of degradation of stable AChRs

L_R = the rate of degradation of RTOs of plus the rate of conversion of RTOs to stable AChRs

t = time (days).

Substituting data from the experiments, and solving by a method that involves iterative curve fitting, we find the following:

1) The curve fits the concept of two exponential rates of degradation very well. The "residual" (or deviation of actual from predicted numbers) is small, indicating a good fit.

2) The true proportion of RTOs can now be calculated.
As expected, it is larger than that derived from extrapolation of the stable curve to 0 time. The difference is attributable to RTOs that are eventually stabilized. The proportion of RTOs equals 21.2% of the total number of receptors in the normal innervated junctions of the SM muscle.

3) Rates for degradation of RTOs and stable AChRs can be calculated, and are:

$\lambda_R = 0.468\ d^{-1}$
$\lambda_S = 0.063\ d^{-1}$

4) The rate for combined degradation plus stabilization of RTOs:

$L^* = 0.702\ d^{-1}$

The most important feature of this model is that it can now be used to examine the stabilization experiment. The equations can be solved to determine what proportion of RTOs should be converted to stable AChRs. This will give us a clear indication of the extent to which denervation prevented the stabilization of RTOs in the studies carried out in Task 2.

Further, using the same considerations, we have developed equations describing the synthesis of AChRs after blockade of pre-existing neuromuscular junctions (Task 1).

The synthesis equation is as follows:

$$N^* = \left[C_R + C_S\right] + \left[\frac{C_S \cdot L_R}{\lambda_S - L_R}\right] e^{-\lambda_S \cdot t} - \left[C_R + \frac{\lambda_S}{\lambda_S - \lambda_R} \cdot C_S\right] e^{-L_R \cdot t}$$

The notations are the same as above.

Task 3. To determine whether depletion of rapidly turning over AChRs results in a deficit of stable AChRs:

This project was completed during year 1, and has now been published (Stanley and Drachman, 1987)

Task 4 (Objective II). To determine the effects of cations on AChR metabolism in vitro:

During year 1 of this project, we completed a study of the effects of the

cations lithium, calcium and sodium on the metabolism of extrajunctional AChRs, using a rat skeletal muscle tissue culture system. These findings were published last year (Pestronk and Drachman, 1987). Our results showed that each of these cations reduced the apparent "synthesis" of extrajunctional AChRs in this system, as measured by ^{125}I-α-BuTx binding.

We now plan to study the mechanism by which these cations down-regulate extrajunctional AChRs. In particular, we will determine whether treatment of cultures with lithium, calcium, and sodium (Drachman et al., 1987; Pestronk 1987) results in a reduction of mRNA for the α- and gamma- subunits of the AChR. Methods for the measurement of mRNAs by hybridization of cDNA probes are described below (Task 4a).

Task 4a: Use of cDNA probes to study regulation of extrajunctional AChR synthesis: measurement of messenger RNAs for receptors.

During the past several years, cDNA probes have become available for the RNA messages for subunits of AChRs of several species (Merlie et al., 1983; LaPolla et al., 1984; Boulter et al., 1985; Mishina et al., 1986; Evans et al., 1987; Parker et al., 1988). These powerful tools enable one to estimate the amount of the relevant mRNA directly, by hybridization techniques (Merlie et al., 1984; Goldman et al., 1985; Klarsfeld and Changeux, 1985; Evans et al., 1987; Moss et al., 1987; Shieh et al., 1987; Parker et al., 1988). These probes offer major advantages in studying certain aspects of regulation of AChR synthesis, including that:

1) Changes in message levels occur promptly, within 24 to 36 hours, well before changes in surface AChRs. This facilitates experiments that can be carried out on only a short-term basis.

2) The changes in appropriate mRNAs are thought to be closer to the level at which regulation of synthesis of AChRs takes place. By contrast, the amount of AChR expressed on the surface membrane is a more remote and indirect effect.

Because of these important advantages, we have devoted a major effort during the past 2 years to acquiring the technology and skills for preparing and using cDNA probes for rodent AChRs. We are applying these methods to projects that are directly germane to the goal of this contract, i.e., understanding the mechanisms of regulation of AChRs:

1) The role of ACh transmission in the regulation of extrajunctional AChR synthesis in vivo

2) The roles of neurotransmitters, cations, and messengers in the regulation of AChR synthesis in vitro.

The first project completed in this category is as follows:

Neural Regulation of mRNA for the α-Subunit of Acetylcholine Receptors: Role of ACh Transmission: Levels of mRNA for AChR subunits are relatively low in innervated skeletal muscles. Following denervation, they rise rapidly, leading to increased AChR synthesis. The mechanism by which motor nerves

normally regulate these mRNA levels is not yet known. The purpose of these experiments is to determine the possible role of ACh transmission in this process. In order to do so, we have compared the effect of surgical denervation with that of blockade of quantal ACh transmission, using botulinum toxin.

Methods:

Experimental procedures were carried out on 180- to 200-gm female Sprague-Dawley rats, under chloral hydrate anesthesia (400 mg/kg).

Denervation: Soleus muscles were denervated by the surgical removal of a 1-cm length of the sciatic nerve in the mid-thigh region. In some experiments, denervation was performed bilaterally; one muscle was used for RNA measurements and the opposite muscle was used for measurement of surface AChRs by 125I-α-BuTx binding.

Botulinum Toxin Treatment: Type A botulinum toxin (generously provided by Dr. E. Schantz), 1.5×10^{-9} gm in 30-μl rat Ringer solution, was injected into the right soleus muscle via a fine 30-gauge needle, on day 0 of each experiment. This produced complete paralysis of the soleus muscle by 3 hours, as determined by stimulating the nerve to the soleus supramaximally and observing the soleus muscle under a stereomicroscope.

Time Course Studies: At time = 0, either denervation or injection of botulinum toxin was carried out. At the subsequent times indicated, from 1 1/2 to 18 days later, soleus muscles were removed whole from anesthetized animals and processed immediately as described below.

RNA Extraction: Total RNA was extracted from individual soleus muscles as follows: The whole muscle was rapidly minced in homogenization medium (50 mM Tris, pH 7.5; 100 mM NaCl; 5 mM EDTA, pH 8.0; 1% socium dodecyl sulfate [SDS]) to a final concentration of approximately 3% (W/V). The suspension was homogenized with the small probe of a Brinkmann Polytron (Westbury, NY) at setting #5, for three 10-second intervals. One-tenth volume of a 2.5 mg/ml solution of Proteinase K (Boehringer Mannheim, Indianapolis, IN) was added, and the mixture was incubated at 37° for 60-90 minutes. This was extracted twice with an equal volume of a 1:1 mixture of neutral Tris-buffered phenol: chloroform/4% isoamyl alcohol. The aqueous supernatant was washed twice with chloroform. RNA was recovered by standard ethanol precipitation (Maniatis et al., 1982) and resuspended in water, and the concentration was determined by measuring the absorbance spectrophotometrically at 260 nm, with an OD of 1.0 corresponding to 40 μg/ml of single-stranded RNA. In order to evaluate the possibility of DNA contamination of these preparations, the RNA was further treated with DNAse on one occasion (Maniatis, 1982), and repurified as above. Recovery was unchanged, indicating that DNA contamination was negligible.

Agarose Gels of RNA and Northern Transfer: RNA was denatured in 50% formamide, 6.6% formaldehyde in "running buffer" (20 mM MOPS, 1 mM EDTA, 5 mM sodium acetate, pH 7.0). The denatured RNA (0-20 μg) was electrophoresed through a 1.5% agarose gel containing 6.6% formaldehyde, in the same buffer. RNA was then transferred to a nitrocellulose filter (Thomas, 1980).

Slot Blots: RNA was denatured in 50% formamide, 6% formaldehyde, 10 mM

Tris, pH 7.4, and transferred to a nitrocellulose filter in a Schleicher & Schuell "Minifold" slot blotting apparatus (Keene, NH).

Preparation of cDNA Probes: α-Subunit cDNA was obtained as an insert of 1720 base pairs from -69 to 1650 in double-stranded M13 vector (generously provided by Dr. J. Merlie, Washington University, St. Louis). Transfection of E. coli JM103, isolation of the RF form and recovery of the cDNA insert by restriction with ECOR1 were carried out as described (Maniatis et al., 1982).

Labeling of cDNA Probes and Hybridization to Filters: The α-subunit probe was labeled with ^{32}P-dCTP, using a nick translation kit from Bethesda Research Laboratories (Bethesda, Maryland). Filters for Northern transfers and slot blots were prehybridized for 2 to 6 hours at 42°C in 50% formamide, 0.1% SDS, 2 mg/ml bovine serum albumin (BSA), 2 mg/ml Ficoll, 2 mg/ml polyvinyl pyrrolidone, 0.1 mg/ml salmon sperm DNA, 0.45M sodium chloride, 45 mM sodium citrate, pH 7.0. Hybridization was carried out for 24 to 48 hours at 42°C in the same solution, except with 0.75 M sodium chloride and 75 mM sodium citrate. Labeled cDNA probe was added to a final concentration of 10^6 cpm/ml. Filters were hybridized for 24 to 48 hours at 42°C, then washed at 65°C for 10 minutes in 0.3M sodium chloride, 30 mM sodium citrate, 0.1% SDS.

RNA Recovery Experiments: In order to evaluate whether experimental differences in mRNA levels might be due merely to differences in RNA recovery in treated and control preparations, a non-muscle ^{3}H-mRNA ("olfactory marker protein message" generously provided by Dr. R. Reed, Johns Hopkins School of Medicine) was added immediately after homogenization of muscle tissue. Samples were taken at each stage of the procedure for control, denervated, and botulinum-injected muscles, and the radioactivity was measured by scintillation counting. No differences in recovery of the labeled mRNA were found in these samples, thereby validating the procedures for recovery of mRNA in differently treated muscles.

Autoradiograms and Scanning Densitometry: Filters were exposed at -70°C to Kodak XAR-5 film with an intensifying screen for 24 hours to 7 days, adjusted so as to attain maximal sensitivity without artifacts due to saturation of the film. The resultant autoradiograms were quantitated using an LKB scanning densitometer. The relative amounts of α-AChR mRNA were expressed in densitometric units per whole muscle. This was calculated on the basis of the total amount of RNA recovered from each muscle, and the amount of RNA from that muscle added to the gel.

Measurement of ACh Receptors: The total number of surface AChRs per muscle was determined by ^{125}I-α-BuTx binding, as previously described (Berg and Hall, 1975; Drachman et al., 1982; Pestronk et al., 1976a,b). Briefly, muscles were teased into three or four pieces, pinned at resting length, and incubated for 4 hours at 37°C in modified Ham's F12 culture medium containing 0.15 μg of ^{125}I-α-BuTx/ml (specific activity, 2 to 5 x 10^4 Ci mol^{-1}). The muscles were washed thoroughly with 12 changes of wash medium, incubated overnight at 4°C in wash medium, and washed again 4 times. The radioactivity due to ^{125}I-α-BuTx bound to the whole soleus muscle was measured in a Micromedics® gamma counter, and expressed as moles x 10^{-15} per whole muscle.

Statistics: Results are given as the mean ± standard error of the mean. The significance of differences between groups was determined by Student's two-

tailed t test.

Results:

Identification of α-Subunit mRNA: We first carried out experiments to validate the specificity of the cDNA probe in our experimental system. Hybridization of the ^{32}P-cDNA probe with Northern blots prepared from agarose gel electrophoresis of total RNA extracts from control, denervated and botulinum toxin-treated muscles revealed a single band at the predicted size for α-AChR mRNA (Buonanno et al., 1986) of two kilobases (Fig. 1). Identification of this band as messenger RNA was further established by: (a) its complete removal after passage over an oligo-d(T) cellulose column, and (b) its recovery from the column with elution buffer (10 mM Tris, pH 7.5; 1 mM EDTA; 0.5% SDS). We also determined that the density of the band on autoradiography of Northern blots was directly proportional to the amount of total RNA applied to the gel. These results indicated that hybridization of the probe to total RNA preparations without gel electrophoresis (i.e., in slot blots) could be used to quantitate α-AChR mRNA. Direct comparison of results obtained from agarose gel electrophoresis and slot blotting were in close agreement.

Effects of Treatment with Botulinum Toxin and Denervation: After botulinum treatment and denervation, both the wet weight of the muscles and the total RNA per muscle decreased progressively throughout the experimental period (Table 1). Because these parameters did not remain constant, we elected not to use them as denominators for AChR-specific mRNA, but instead expressed α-AChR mRNA per whole soleus muscle.

The control innervated muscles had low levels of α-AChR mRNA, requiring longer exposure times than those for denervated or botulinum-treated muscles to detect the hybridization bands on autoradiograms (Fig. 1). Following denervation, the α-AChR mRNA increased significantly within 1 1/2 days ($p < 0.001$), and reached a maximal level approximately 15 times that of the control innervated muscles by 2 1/2 days (Fig. 2). This high level was maintained throughout the 7 1/2-day course of the experiment. Botulinum-treated muscles also showed a significant increase in α-AChR mRNA by 1 1/2 days ($p < 0.001$), but the rate of rise was more gradual. The levels in α-AChR mRNA in the botulinum-treated muscles were significantly less than those of the denervated muscles at the earlier 1 1/2 and 2 1/2-day time points ($p < 0.01$), but eventually reached values equal to those of denervated muscles by 7 1/2 days.

Changes in the surface AChRs of denervated and botulinum-treated muscles were similar to, but more gradual than, those of the α-AChR mRNA (Fig. 3). Following denervation, surface AChRs per muscle, as measured by ^{125}I-α-BuTx binding, first increased significantly by 2 1/2 days ($p < 0.01$), and reached a plateau at 3 1/2 days. The increase in surface AChRs after botulinum injection was slower, reaching a peak at 5 1/2 to 7 1/2 days.

Recovery after Botulinum Treatment : It is well established that both ACh blockade and the denervation-like effects of botulinum toxin eventually recover, unless the injections are repeated (Bambrick and Gordon, 1987). To determine whether the increase of α-AChR mRNA is similarly reversible after a single injection of botulinum toxin, we carried out hybridization experiments at intervals up to 18 days after treatment. As shown in Fig. 4, α-AChR mRNA initially increased in botulinum-treated muscles, but then decreased

significantly ($p < 0.02$) by 18 days. In contrast, α-AChR mRNA levels in surgically denervated muscles remained at the high plateau values ($p > 0.1$).

Interpretation and Discussion:

Our findings indicated that denervation results in an increase of mRNA for the α-subunit of AChR, which begins promptly (before 36 hours) (Merlie et al., 1984; Goldman et al., 1985; Evans et al., 1987; and Moss et al., 1987).

Blockade of quantal ACh release, by means of botulinum toxin produced a denervation-like increase in mRNA for the α-subunit of AChR. However, the rise was significantly slower, first reaching a maximum at 7 1/2 days. At a later time (18 days), as the ACh-blocking effects of botulinum toxin wore off, the mRNA levels fell significantly, in contrast to the sustained maximum level in surgically denervated muscles.

The effects of denervation and botulinum toxin on surface AChRs paralleled the effect on the mRNAs, but occurred later (beginning at 2 1/2 days) and were somewhat less marked (maximum-5 to 6-fold increase over the controls). In addition, we confirmed the results of previous experiements showing that the increase of surface AChRs occurred more slowly after botulinum treatment than after denervation. These findings indicate that the effect of botulinum toxin is similar, but not quantitatively equivalent, to that of denervation.

Interpretation of these results depends on an understanding of the action of botulinum toxin. Botulinum inhibits the quantal release of ACh from cholinergic nerves (Simpson, 1981; Stanley and Drachman, 1983b). It has no other known actions on nerves or muscles, and in particular does not produce structural damage nor interfere with axonal transport (Thesleff and Sellin, 1980; Pestronk et al., 1976a). The most straightforward interpretation of our findings is that the known effect of botulinum toxin in blocking quantal ACh transmission is responsible for the denervation-like increase of mRNA for the α-subunit of AChR. This suggests that quantal ACh transmission normally plays an important role in mediating the motor nerve's regulatory effect on mRNA for AChR. However, the effect of botulinum treatment was not quantitatively equivalent to that of surgical denervation. The slower time course of the increase of mRNA after botulinum treatment as compared with denervation cannot be attributed to a delay in the blocking action of the toxin, since complete paralysis and cessation of miniature endplate potentials occur within a few hours after injection of botulinum toxin (Pestronk et al., 1976a). Clearly, botulinum toxin does not eliminate the entire regulatory influence of the nerve, suggesting that some factor in addition to quantal ACh transmission and muscle activity is also involved. We have previously reported that botulinum toxin does not block the substantial release of ACh that occurs by a non-quantal mechanism (Stanley and Drachman, 1983b), and have also shown that this component of ACh transmission can account for the remaining neural influence in regulating certain properties of muscles including extrajunctional surface AChRs (Drachman et al., 1982). This strongly suggests that the persistence of non-quantal ACh transmission may also account for the difference between the effects of botulinum toxin and denervation on the increase of mRNA for the α-subunit of AChR.

Now that we have established the method for perfusion of the soleus muscle with α-BuTx, we will test the effect of blockade of quantal plus non-quantal ACh

transmission, using this agent. We anticipate that α-BuTx blockade should produce an effect that is quantitatively equivalent to the effect of denervation.

Task 7. To determine whether ACh transmission plays a role in maintenance of stability of junctional AChRs:

The majority of AChRs at normally innervated neuromuscular junctions are stable, with a half-life averaging about 12 days in most rodent muscles (Berg and Hall, 1975; Stanley and Drachman, 1983a; Bevan and Steinbach, 1983). Following denervation, the rate of degradation of pre-existing junctional AChRs becomes accelerated, after a lag period (Stanley and Drachman, 1981; Bevan and Steinbach, 1983; Salpeter and Loring, 1985). The mechanism by which the nerve normally maintains stability of the junctional AChRs is not yet known. These experiments were designed to determine whether ACh transmission plays a role in maintenance of the stability of junctional AChRs. Our findings (see below) indicate that neuromuscular ACh transmission plays a key if not exclusive role in mediating the nerve's effect on stability of junctional AChRs.

Female Swiss mice were used throughout these experiments, and were anesthetized with chloral hydrate (0.5 gm/kg body weight) and ether for all procedures.

Labeling of AChRs with ^{125}I-α-BuTx: AChRs of soleus muscles were labeled by injecting approximately 1 μg per muscle 125I-α-BuTx (0.04 μg/gm body weight) in 10 μl phosphate-buffered saline under direct visualization. In the experiment on the flexor digitorum brevis muscles, injections were made percutaneously into the sole of the right foot (0.5 μg ^{125}I-α-BuTx in 20 μl of phosphate-buffered saline. These doses of ^{125}I-α-BuTx have been shown to label >99% of AChRs (Ramsay et al., 1988). The binding of ^{125}I-α-BuTx is virtually irreversible, with less than 3% dissociation per day (Ramsay et al., 1988; Bevan and Steinbach, 1983; Berg and Hall, 1975b). It has previously been shown that labeling AChRs with ^{125}I-α-BuTx does not alter their turnover significantly (Devreotes and Fambrough, 1975).

Preparation of Muscles with Pure Populations of Labeled Stable AChRs: In order to follow the turnover of a pure population of stable receptors, we waited 6 days after labeling the junctional AChRs, so as to allow virtually all the RTOs to be degraded (Stanley and Drachman, 1983a,1987; Ramsay et al., 1988). At this time point (designated day 0) the only remaining labeled AChRs are the stable ones.

Surgical Denervation: The soleus and flexor digiturum breis muscles were denervated on day 0 by sectioning the sciatic nerve in the mid-thigh and avulsing its proximal end to avoid reinnervation.

Presynaptic Blockade with Botulinum Toxin: Purified Type A botulinum toxin (generously provided by Dr. E. Schantz) was freshly diluted in mammalian Ringer solution before use. In the soleus muscle, injections were made under direct visualization (1.5×10^{-10} gm on day 0, and 1.0×10^{-10} gm on days 6 and 12). In the flexor digitorum brevis muscle, 1.5×10^{-10} gm of botulinum toxin was injected percutaneously on day 0, and 1.0×10^{-10} gm was injected on days 5, 12 and 19. All control animals received injections of Ringer solution at the same times. A volume of 10 μl was used for all injections.

Postsynaptic Blockade with α-BuTx: Continuous neuromuscular blockade was produced by perfusing soleus muscles with purified α-BuTx via implantable osmotic pumps as previously described (Drachman et al, 1982; Pestronk and Drachman, 1985). Neuromuscular blockade was initiated at day 0 by injecting 1.1 μg of α-BuTx in phosphate-buffered saline solution directly into the muscle via a fine 30-gauge needle. Blockade was then maintained by continuous perfusion of α-BuTx in phosphate-buffered saline solution (0.06 μg/μl at 0.46 μl/hour) by means of Alzet model 2002 mini-osmotic infusion pumps (Alza Corp., Palo Alto, CA). The pumps were implanted subcutaneously into the back and the solution was delivered directly over the belly of the soleus muscle via tapered PE60 polyethylene tubing (Clay Adams, Parsippany, NJ) sutured in place. Control animals were injected and perfused in the same manner with similar volumes of PBS solution. The number of infusion pumps that could be implanted in a single experiment was limited, and we therefore elected to study the 2 time points -- i.e., 4 and 6 days -- which were found to be critical in our denervation and botulinum experiments. After 4 and 6 days of treatment, the muscles were removed and the loss of stable junctional AChRs was evaluated as described below (see Results).

Measurement of Turnover of Stable Junctional AChRs: A baseline was established by removing muscles with labeled stable AChRs from the control group on day 0. Samples of 4-13 muscles were removed from the control and experimental groups at various times thereafter. Radioactivity bound to junctional AChRs was measured by counting the whole excised muscle in a gamma spectrometer (Micromedic Systems, Inc., Horsham, PA). The counts for each muscle were corrected for decay, and then expressed as a fraction of the mean counts of the baseline (day 0) control group for that batch of experimental mice.

In these experiments, ^{125}I-α-BuTx bound to the whole soleus or flexor digitorum brevis muscle was used as a measure of radioactivity bound to the junctional AChRs. We conducted experiments which showed that at 6 days or more after labeling, the nonspecific or extrajunctional binding was negligible. To assess the degree of nonspecific binding, we washed the muscles extensively (18 washes over 18 hours). This removed less than 1% of the bound radioactivity . In order to assess the degree of extrajunctional binding, we measured the radioactivity in the junctional region relative to extrajunctional regions. We found that 6 days after labeling more than 98% of the total muscle radioactivity was bound at the junctional regions. Thus whole muscle radioactivity can be used as an accurate measure of junctional radioactivity.

Analysis of Data: The data for each muscle (soleus or flexor digitorum brevis) at each time point were pooled; means and standard errors of the means were calculated, and in the experiments with sufficient numbers of time points, were plotted on a semi-log graph by the least squares method. Data for groups of muscles were compared by Student's two-tailed t-test.

Results: The results of this study show that treatment of the mouse soleus and flexor digitorum brevis muscles with botulinum toxin produced a significant denervation-like acceleration of degradation of labeled stable junctional AChRs, although the onset of this effect was delayed as compared with that of surgical denervation. By contrast, postsynaptic blockade with α-BuTx, which blocks both quantal and non-quantal ACh transmission, produced an effect which was

quantitatively equivalent to that of denervation, with an identical time course.

Presynaptic Blockade of Neuromuscular Transmission with Botulinum Toxin: Figure 5 shows the degradation curves for stable junctional AChRs of the soleus muscle. In the control muscles, bound radioactivity was lost with a half-life of approximately 11.5 days. Within 4 days after denervation, there was a significantly greater loss of junctional AChRs as compared to the controls ($p < 0.01$), and an increase in the rate of degradation, with a half-life of 3.6 days. Following treatment with botulinum toxin, the loss of AChRs first became significantly different from controls at 6 days (i.e., 2 days later than in the denervated group) ($p < 0.01$). The rate of degradation increased, after botulinum treatment, to a half-life of 3.6 days. However, at the 6-day time point, the loss of labeled junctional AChRs in botulinum-treated muscles was not as great as that in the denervated muscles at 6 days ($p < 0.01$).

Figure 6 shows the degradation curves for stable junctional receptors of the FDB muscle. In the control muscles, radioactivity was lost with a half-life of 16.2 days. This rate of degradation was consistently slower than that of the junctional AChRs of the soleus muscle measured here, and the reported rate for the sternomastoid muscles (Salpeter and Loring, 1985; Ramsay et al., 1988). Following denervation, there was a trend toward more rapid degradation of AChRs by day 16, but the difference between denervated and control muscles first became statistically significant at day 20 ($p < 0.001$). The half-life of junctional AChRs at this time was 4.5 days. Botulinum-treated muscles also showed a greater loss of labeled junctional AChRs, which first reached statistical significance later, at day 29 ($p < 0.01$).

As noted above, the accelerated turnover of AChRs began earlier after denervation than after botulinum treatment; in the soleus muscle, the loss of stable junctional AChRs was significantly greater at 4 days and 6 days ($p < 0.01$).

Postsynaptic Blockade of ACh Transmission with α-bungarotoxin: In order to compare the effects of postsynaptic blockade of ACh transmission with those of denervation, we measured junctional AChRs of soleus muscles at 4 and 6 days after the beginning of α-BuTx treatment and denervation. Table 1 shows that both procedures produced a highly significant loss of junctional AChRs at 4 and 6 days, as compared to controls ($p < 0.01$). Most important, the effects of α-BuTx treatment did not differ from those of denervation at either time point ($p > 0.1$).

Interpretation: This investigation was designed to examine the role of neuromuscular synaptic transmission in the maintenance of stability of junctional AChRs. Our findings showed that botulinum toxin, which blocks quantal ACh transmission, and α-BuTx, which blocks both quantal and non-quantal ACh transmission, resulted in "destabilization" of pre-existing AChRs at neuromuscular junctions, similar to the effects of denervation. The only known common denominator of action of botulinum toxin and α-BuTx is interference with ACh transmission. Therefore, the most straightforward interpretation of our findings is that blockade of ACh transmission is responsible for the denervation-like destabilization of junctional AChRs. This suggests that cholinergic neuromuscular transmission normally plays a major, if not exclusive, role in mediating the motor nerve's effect on stability of junctional AChRs.

The delayed onset of increased AChR turnover after botulinum treatment, as compared with denervation or α-BuTx treatment, was a consistent finding in our experiments. This cannot be attributed to a delay in the cholinergic blocking action of botulinum toxin, since complete paralysis and nearly complete cessation of miniature endplate potentials occur within a few hours after injection of the toxin (Kao et al., 1976; Pestronk et al., 1976a). We favor the interpretation that the failure of botulinum toxin to block the spontaneous non-quantal release of ACH (Stanley and Drachman, 1983b) may account for the difference between its effects and those of denervation or α-BuTx treatment. This is consistent with previous observations that non-quantal ACh transmission has a significant partial influence in regulating certain other properties of muscles (Mathers and Thesleff, 1978; Drachman et al., 1982).

The mechanism by which ACh transmission influences the stability of junctional AChRs is not yet known. Clearly, the effects of ACh cannot be exerted directly on the AChRs whose turnover is being followed in the experiments, since the ^{125}I-α-BuTx used as a label itself blocks the ligand-binding sites of these AChRs. However, ACh transmission acting on non-blocked AChRs at the same junctions presumably is responsible for mediating a stabilizing effect. The differences between stable and destabilized AChRs have been defined only in terms of their turnover times. However, this undoubtedly reflects biochemical or structural differences between them. Possible differences include covalent modifications of the AChR molecules (reviewed in Salpeter and Loring, 1985), attachment of AChRs to cytoskeletal elements (Bloch and Hall, 1983; Froehner, 1986), or alterations in surrounding microenvironment of the synaptic membrane (McMahan et al., 1984).

Perhaps the most important biological implication of the present work is its possible relation to synaptic memory processes. Previous studies of synaptic memory have focused for the most part on presynaptic mechanisms that modify neurotransmission (Kandel et al., 1987). By contrast, stabilization of AChRs constitutes a long-term modification of the neuromuscular junction at a postsynaptic level. AChR stabilization takes place over a much more extended time scale than the phenomenon of receptor "desensitization," which has previously been proposed as a form of short-term postsynaptic memory (Changeux et al., 1984,1987). Thus AChR stabilization represents a model of long-term postsynaptic "memory," albeit at a peripheral synapse between motor nerve and skeletal muscle cells. The present results demonstrate that neurotransmission plays a key role in mediating this process. We suggest that similar mechanisms of transmitter-driven receptor stabilization may be involved in memory processes in the central nervous system as well.

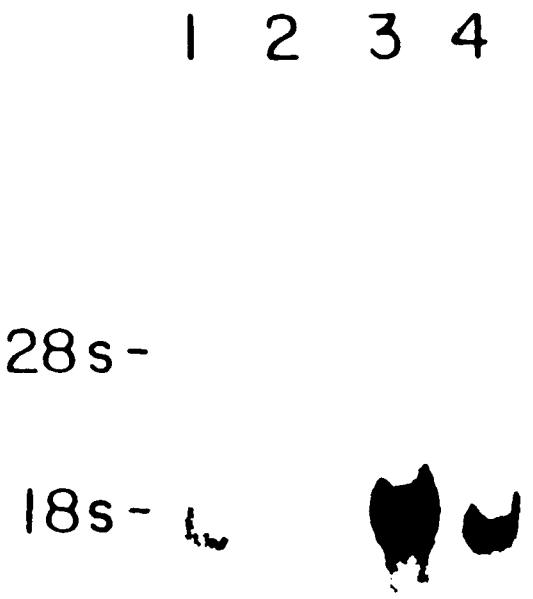

Figure 1. Identification of α-Subunit mRNA by Northern Blots.

^{32}P-cDNA for the α-subunit was hybridized after agarose gel electrophoresis of total muscle RNA and transfer to nitrocellulose filters. The autoradiographic exposure was 7 days for lane 1 only, 24 hrs. for lanes 2-4. Lane 1: innervated control muscle (20 μg RNA). Lane 2: same as lane 1, but note difference in autoradiographic exposure. Lane 3: 4 days post-denervation (8.7 μg RNA). Lane 4: 4 days post-botulinum treatment (8.3 μg RNA). Approximate positions of 18S and 28S ribosomal RNA markers are indicated. All 3 bands were in the appropriate position for the α-subunit of AChR. Visualization of the mRNA in the innervated muscle required far longer exposure.

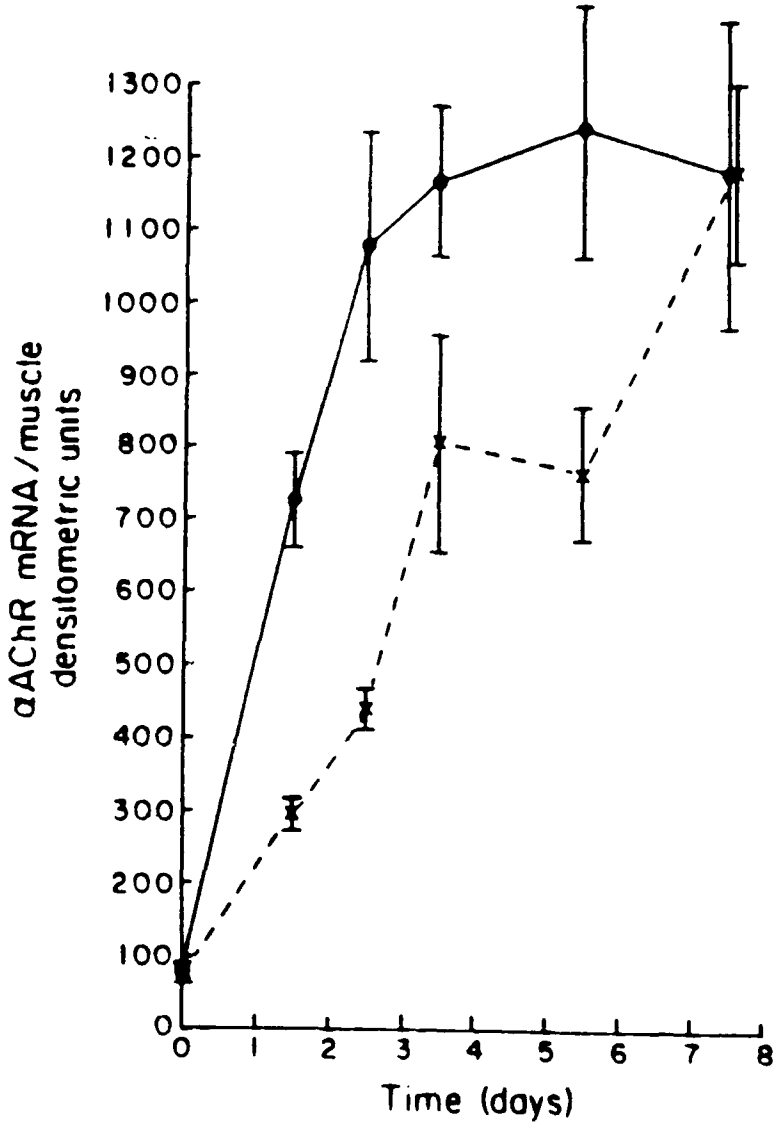

Figure 2. Effects of Botulinum Treatment and Denervation on α-AChR mRNA Levels.

α-AChR mRNA was estimated by densitometer scans of autoradiograms obtained after hybridization of ^{32}P-cDNA for the α-subunit to slot blots of muscle RNA, and are expressed as arbitrary units per whole soleus muscle (for details, see text). Values are means ± SEM for 4-5 muscles in each group. ●——● = denervated muscles; X- - -X = botulinum-treated muscles. Note the lower values for botulinum-treated muscles, reaching equivalent levels, approximately 15 x controls, by day 7 1/2.

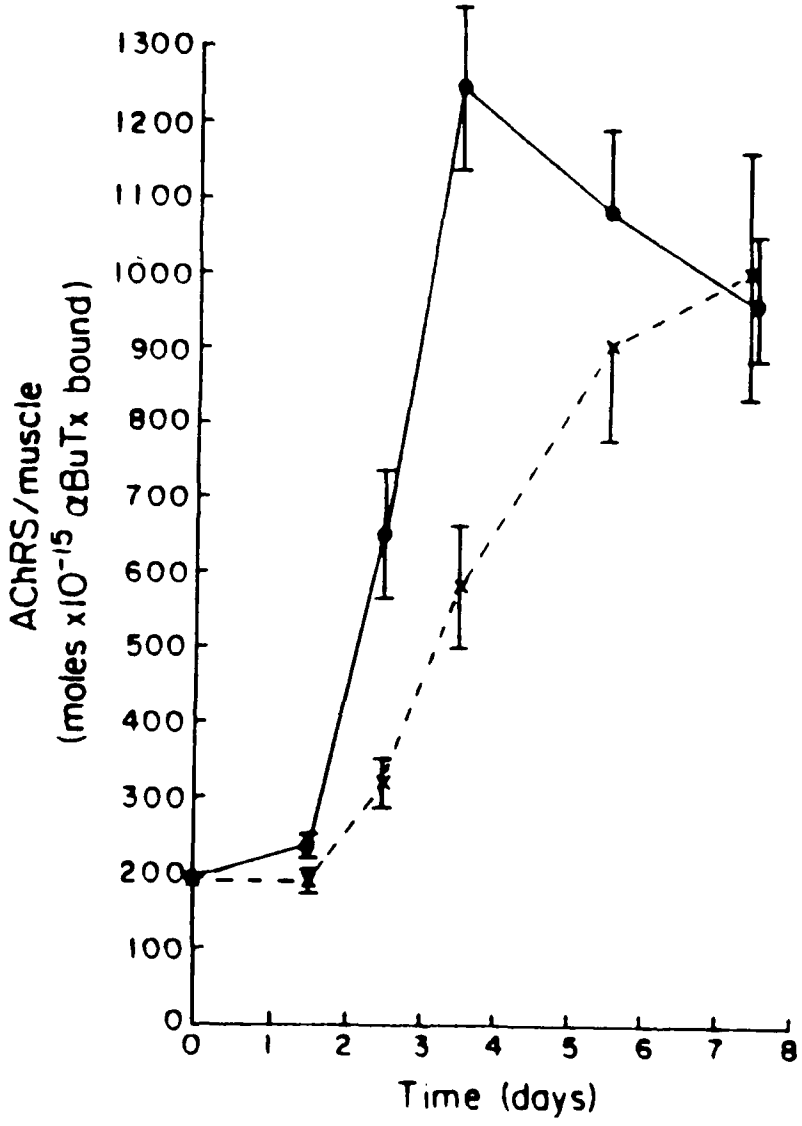

Figure 3. Effects of Botulinum Treatment and Denervation on Surface AChRs.

Surface AChRs were measured by binding of ^{125}I-α-BuTx, and expressed as moles α-BuTx bound per whole soleus muscle. ●——● = denervated muscles; X- - -X = botulinum-treated muscles. Note that surface AChRs rise later than α-AChR mRNA (cf Fig. 2), and that the level of surface AChRs in botulinum-treated muscles is lower than for denervated muscles at early time points, later reaching equivalence.

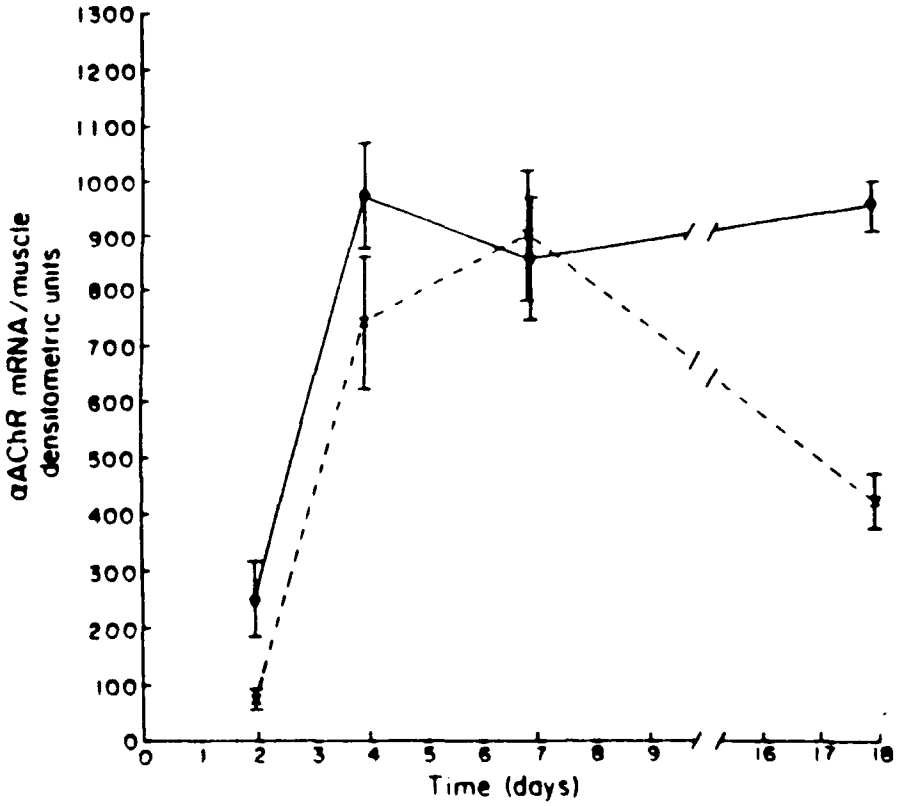

Figure 4. Effects of Botulinum Treatment and Denervation on α-AChR mRNA Levels at Early and Late Time Points.

α-AChR mRNA was estimated by densitometer scans of autoradiograms obtained after hybridization of ^{32}P-cDNA for the α-subunit to Northern blots of muscle RNA, and are expressed as arbitrary units per whole soleus muscle (see text). Values are means ± SEM for 3-4 muscles in each group. ●——● = denervated muscles; X- - -X = botulinum-treated muscles. Note the initially lower values for botulinum-treated muscles, reaching equivalence by 7 days, and decreasing significantly by 18 days with recovery from effects of botulinum toxin.

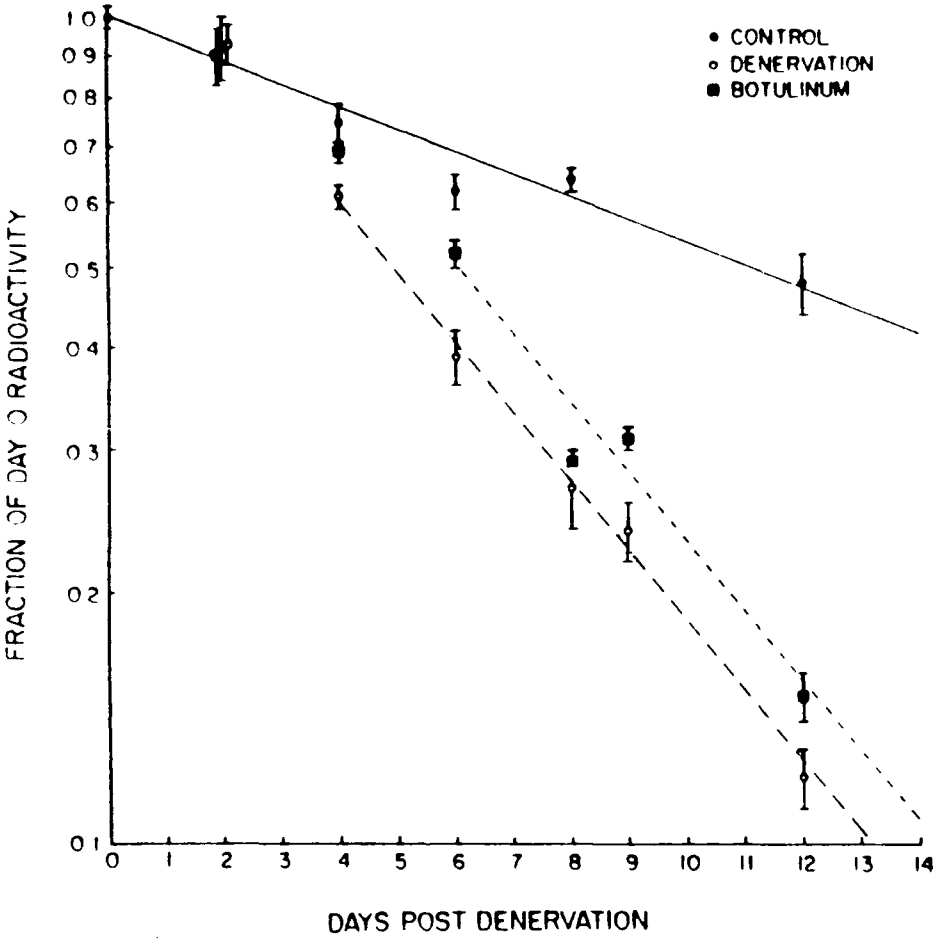

Figure 5. Effects of Surgical Denervation and Botulinum Toxin Treatment on Turnover of Stable Junctional AChRs in the Soleus Muscle.

Soleus muscles of mice were either denervated, treated with botulinum toxin, or treated with control injections, as described. The number of muscles at each time point, with each treatment, varied from 6 to 19. Lines were plotted by the least squares method. Note the accelerated loss of junctional AChRs in the denervated and botulinum-treated muscles. The difference between control and denervated muscles was significant at day 4 ($p < 0.01$); for botulinum-treated muscles, the difference first became significant at day 6 ($p < 0.01$). Error bars represent standard error of the means.

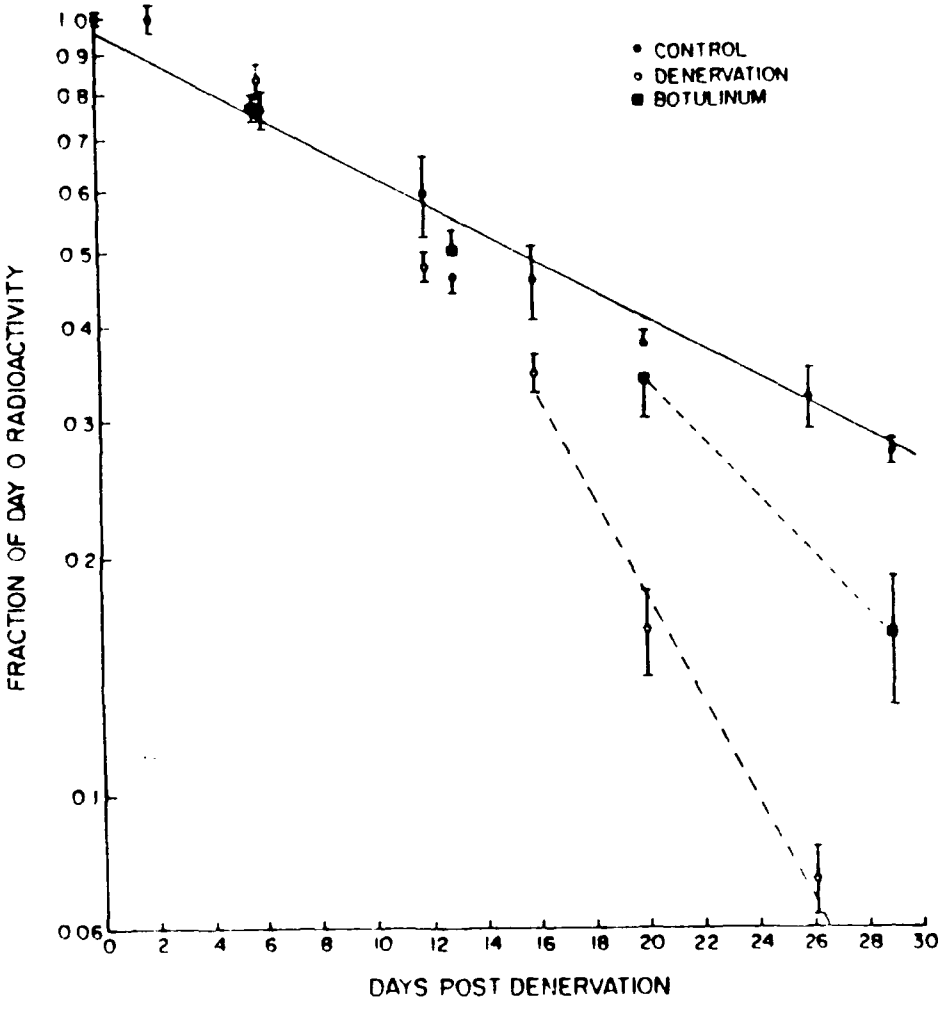

Figure 6. Effect of Surgical Denervation and Botulinum Toxin Treatment on Turnover of Stable Junctional AChRs in the Flexor Digitorum Brevis Muscle.

Flexor digitorum brevis muscles of mice were treated as in Fig. 5. The number of muscles at each time piont, with each treatment, varied from 4 to 12. Lines were plotted by the least squares method. Note the accelerated loss of junctional AChRs in the denervated muscles, reaching significance at day 20, and in the botulinum-treated muscles at day 29. Error bars represent standard error of the means.

Table 1

Effects of Denervation and Botulinum Toxin on Wet Weight and RNA Content of Soleus Muscles

(A) Wet Weight of Soleus Muscle (mg) After Varius Treatments

Treatment	Time (Days) 0	1.5	2.5	3.5	5.5	7.5
Control	103 ± 2.9					
Denervated		102.5 ± 3.2	87.9 ± 2.9	84.4 ± 3.8	75.0 ± 6.2	70.7 ± 5.6
Botulinum		98.8 ± 4.0	90 ± 3.3	82.3 ± 3.7	73.3 ± 3.4	64.8 ± 3.2

(B) Total RNA (μg) per Soleus Muscle After Various Treatments

Treatment	Time (Days) 0	1.5	2.5	3.5	5.5	7.5
Control	179 ± 17.5					
Denervated		177 ± 8.5	145 ± 5	121 ± 7	114 ± 8	126 ± 6.2
Botulinum		188 ± 12	160 ± 6	139 ± 12	124 ± 14	118 ± 10

Effects of botulinum treatment and denervation on (A) wet weight (mg) and (B) total RNA content (μg) of soleus muscles. The number of muscles used for wet weights was 10 for each time point for denervation and botulinum toxin treatment, and 4 for the control group. Total RNA was determined using groups of 4 or 5 muscles at each time point for each treatment. Both wet weight and total RNA per muscle decreased with time after treatment; there were no significant differences between the effects of the two treatments with respect to either parameter. Each muscle used in this table came from an individual animal; no muscle pairs were used.

Table 2

Rapid Loss of Junctional AChRs Following
α-BuTx Treatment or Denervation

Treatment	Experimental Period: 4 days	6 days
Ringer infusion (controls)	0.84 ± 0.03 (N = 6)	0.70 ± 0.05 (N = 5)
α-Bungarotoxin infusion	0.59 ± 0.04 (N = 7)[a,b]	0.48 ± 0.03 (N = 11)[a,b]
Denervation	0.64 ± 0.02 (N = 7)[a]	0.46 ± 0.02 (N = 6)[a]

The proportion of junctional AChRs remaining at each time point is expressed as the mean fraction of the day 0 control values ± SEM. Note the loss of junctional AChRs in α-BuTx-treated and denervated muscles as compared with Ringer controls.

[a] - Less than control; $p < 0.01$.
[b] - Not different from denervation; $p > 0.1$.
N = Number of animals.

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